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Detection of Biomarkers with Solid-Phase Proximity Ligation Assay in Patients with Colorectal Cancer

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Abstract

BACKGROUND: In the search for prognostic biomarkers, a significant amount of precious biobanked blood samples is needed for conventional analyses. Solid-phase proximity ligation assay (SP-PLA) is an analytic method with the ability to analyze many proteins at the same time in small amounts of plasma. The aim of this study was to explore the potential use of SP-PLA for biomarker validation in patients with colorectal cancer (CRC). *MATERIAL AND METHODS:* Plasma samples from patients with stage I to IV CRC, with ($n = 31$) and without ($n = 29$) disease dissemination at diagnosis or later, were analyzed with SP-PLA using 35 antibodies targeting an equal number of proteins in 5-μl plasma samples. Carcinoembryonic antigen (CEA), analyzed earlier in this cohort using a different technology, was used as a reference. *RESULTS:* A total of 21 of the 35 investigated proteins were detectable with SP-PLA. Patients in stage II to III with disseminated disease had lower plasma concentrations of HCC-4 ($P = .025$). Low plasma levels of tissue inhibitor of metalloproteinases–1 were seen in patients with disseminated disease stage II ($P = .003$). The level of CEA was higher in patients with disease dissemination compared with those without ($P = .007$). *CONCLUSION:* SP-PLA has the ability to analyze many protein markers simultaneously in a small amount of blood. However, none of the markers selected for the present SP-PLA analyses gave better prognostic information than CEA.

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Introduction

Tumor markers in colorectal cancer (CRC) have the potential to play a crucial role in screening, prognostication, and therapeutic monitoring. Carcinoembryonic antigen (CEA) is the most known and widely used marker. Because of its low sensitivity for identifying individuals with CRC, CEA is not recommended for screening [1]. Even though CEA has low sensitivity, it is the only marker certificated for detection of early recurrence, although many patients with tumor relapse have normal CEA levels [2].

Earlier survival analyses in the present CRC cohort revealed that patients with stage I disease have no risk of developing tumor recurrence for up to 5 years, although the 5-year overall survival (OS) was only 75% due to death from other diseases. In CRC stage II, only 14% developed cancer recurrence, and the 5-year OS was similar to stage I or 74%. In CRC stage III, 40% developed disease recurrence, and the 5-year OS was 54% [3]. For stage III CRC patients, adjuvant treatment improves OS by 15% to 20%, whereas 60% are already cured by the primary surgery alone [4]. The use of adjuvant therapy is not routinely recommended for patients with CRC stage II, but for groups at high risk for recurrence like those with T4, emergency surgery and analysis of a few lymph nodes may be offered treatment, although the benefits are not well proven [5].

It would certainly be of great benefit to find biomarkers that could identify those 14% of stage II and 40% with stage III CRC who will have cancer recurrence. This could radically change the strategy of

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adjuvant chemotherapy use, sparing those not in need of the treatment with all its side effects and improving the surveillance of those at higher risk of tumor recurrence.

The proximity ligation assay (PLA) is a recently described protein detection assay. Pairs of oligonucleotide-labeled antibodies (PLA-probes) are used to detect the target antigen. When two such PLA-probes bind the same antigen, the probes are brought in proximity, leading to formation of a template DNA strand by ligation. The DNA strand is then amplified by quantitative real-time polymerase chain reaction to detectable signals [6]. Solid- phase PLA (SP-PLA) is a form of PLA where antibodies immobilized on a solid support act as capture agents for the target proteins before the PLA [7]. There are several advantages in detecting plasma biomarkers using PLA, such as increased specificity, minimal sample consumption, and the capacity to simultaneously analyze numerous targets in a multiplex format [8].

The primary aim of this study was to explore the use of SP-PLA to evaluate the concentrations of a set of potential biomarkers in clinical plasma samples from patients with CRC according to disease stage and recurrence in relation to CEA. For this purpose, a strategic sample of a limited number of patients was selected.

Material and Methods

Sixty patients from a previously described cohort of 320 patients [9], operated for CRC at the Central District Hospital, Västerås, County of Västmanland, were strategically selected. Nine patients had disease stage I, one of whom had a recurrence after 7 years. Twenty-two patients had stage II disease, 10 of whom had a recurrence; 19 had stage III disease, with 9 having a recurrence; and 10 patients were at stage IV at diagnosis. Patients were divided into two groups: those with disease dissemination (stage I-III with recurrence and stage IV) and those without dissemination (stage I-III without recurrence). The purpose was to select approximately 10 patients each with stage I, stage II with/without dissemination, stage III with/without dissemination, and stage IV. Among patients with nondisseminated disease, six received postoperative adjuvant chemotherapy of which all were in stage III of the disease. The patients were treated between August 2000 and December 2003. Information about stage, localization, differentiation, and vascular and neural invasion was received from pathology records. Information on death and cancer recurrence was received from surgical and oncology records and from the Clinical Database for Colorectal Cancer held at the Regional Oncologic Centre in Uppsala/Örebro.

Preoperative collection of blood samples was drawn into EDTA tubes and processed for plasma by centrifugation. The samples have been stored in −70°C for at least 10 years before being analyzed.

Methods

SP-PLA. From each patient, 5 μl of plasma was used for protein detection with multiplex SP-PLA, as described by Darmanis [6,7]. The multiplex protein detection panel was preselected by the science group of U Landegren for explorative studies and comprised 35 proteins previously reported as biomarkers for cancer, inflammation, or cardiovascular disease, and 1 internal control (mouse IgG) [6]. For each protein in the panel, individual dilutions of recombinant proteins were prepared at high, medium, or low concentrations (1 nM, 10 pM, and 0.1 pM, total volume of 45 μl). Quantification of each protein was made by real-time polymerase chain reaction of the DNA reporter strands that formed in the detection reactions. Molar protein concentrations were converted to pg/μl before analysis.

Statistical Analyses

The χ^2 and Fisher's exact tests were used for comparisons of categorical variables. The Mann-Whitney *U* test was used to compare the plasma levels of selected biomarkers and dissemination status and between each disease stage. Kruskal-Wallis test was used to compare values of plasma levels biomarkers and stage of the disease.

Statistical significance was set at $P < .05$. All observations were censored at the end of the study period (15th April 2010).

SPSS statistics version 21 (SPSS Inc., Chicago, IL) was used for statistical analysis.

Ethical approval (number 2000:001) was obtained from the Ethics Committee at Uppsala University, Uppsala, Sweden.

Results

Patient Characteristics

Of 60 patients, 65% had colon cancer; and 35%, rectal cancer. Thirty-one patients had disseminated disease, and 29 were without dissemination. The median age of patients with dissemination was 69 years (range, 34-85), and for patients with nondisseminated disease, it was 76 years (range, 49-91) (Table 1). There were no statistically significant differences in age, gender, tumor localization, tumor differentiation, and presence of vascular and neural invasion between the two groups. The cases with vascular or neural invasion were, however, confined to the disseminated group. Patients with disease dissemination had higher CEA levels than those without ($P = .007$) (Table 1), which could also be seen when comparing the disease stages ($P = .040$) (Table 2).

Plasma Analyses of Detectable Biomarkers

The plasma level of 35 biomarkers (Table 3), of which 21 were detectable, and also including one internal control were analyzed with

Table 1. Comparison of Clinicopathological Characteristics in Patients with and without Disease Dissemination

Characteristics	No Dissemination (*n* = 29)	Dissemination (*n* = 31)	*P* Value
Age (years)			
Median (range)	76 (49-91)	69 (34-85)	NS
Gender			
Female	13	18	NS
Male	16	13	
Localization			
Right/left colon	14/15	9/22	NS
Colon/rectum	20/9	19/12	
Disease stage			
I	8	1	*
II	12	10	
III	9	10	
IV	-	10	
Differentiation			
Well-moderate	26	25	NS
Poor	3	9	
Mucinous			
Yes	3	4	NS
No	26	26	
Vascular invasion			
Yes	0	5	NS
No	29	25	
Neural invasion			
Yes	0	2	NS
No	29	28	
CEA			
<6 ng/ml	24	15	.007
≥6 ng/ml	5	16	

* Not relevant testing due to strategic selection of patients.